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Ethyl-carbamate determination by gas chromatography–mass spectrometry at different stages of production of a traditional Brazilian spirit

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ABSTRACT

Ethyl carbamate (EC), which is probably carcinogenic to humans, can be produced during the alcoholic fermentation of sugar-cane juice to give *cachaça*. The stages to produce *cachaça* are obtainment of sugar-cane juice, sugar-cane fermentation to wine, and obtainment of distilled fractions and residue. In order to investigate the presence of EC in the wine and in the fractions of the distillation process, as well as in the vinasse (the residue left after distillation), gas chromatography–mass spectrometry was employed. After the fermentation phase, the wine showed an average content of 122 mg L^{-1} of EC. Average EC content in distilled fractions was 59.7 mg L^{-1} for head, 52 μg·L^{-1} for heart and 1.57 mg L^{-1} for tail. EC content was 53.1 mg L^{-1} for vinasse. The results showed that it is essential to separate the head and tail fractions to ensure *cachaça* quality, with respect to EC content.

1. Introduction

Brazil is known for producing distilled alcoholic beverages from sugar-cane juice fermentation. *Cachaça* and other cane juice spirits are some of the beverages produced and appreciated in Brazil as well as in many countries around the world. Brazil's annual *cachaça* production is estimated at 1.8×10^9 L. A significant amount is exported (Bruno, 2006).

Cachaça is a typical sugar-cane spirit produced exclusively in Brazil, obtained by the distillation of fermented sugar-cane juice, with unique sensorial characteristics, to which sugar, as sucrose, may be added at up to 6 g per litre. Brazilian standards establish that this beverage should have an alcoholic content of 38–54% (v/v), at 20 °C, obtained by lowering the alcohol concentration of the simple distillate by water addition or by distillation of simple fermented sugar-cane juice (Brasil, 2005).

The production of *cachaça* can be described according to the following steps: the sugar-cane is harvested, transported and received in the processing plant, and ground. The sugar-cane juice obtained is decanted and diluted to 15 °Brix. Then it is fermented and subsequently distilled to separate the fractions. The distillation process, in traditional *cachaça* production, produces three fractions called head, heart, and tail, corresponding to the order in which they leave the alembic during the distillation process. *Cachaça* is distilled in copper retorts and copper contamination can take place. The producers consider that distillation in copper apparatus is, however, necessary to guarantee good sensorial properties in the product, due to the catalytic effects of the element on the formation of flavour (Neves, Oliveira, Fernades, & Nobrega, 2007). The replacement of copper by stainless steel in the retorts, in order to eliminate this contamination, has a negative effect on the sensory quality of this beverage, as shown by several previous studies (Faria, Franco, & Piggott, 2004; Faria & Pourchet Campos, 1989; Faria, Deliza, & Rossi, 1993; Isique, Cardello, & Faria, 1998). On the other hand, the toxic effects of high concentrations of copper have been investigated, as well as the determination of this element in beverages, since an excess of copper in alcoholic beverages can cause serious damage to health (Goyer & Cherian, 1995). *Cachaça*, by definition, is the heart fraction of the distillation and can be stored in wooden or stainless steel vessels, to rest, after which it is bottled (Cardoso, 2006).

In the alcoholic fermentation of cane juice, sugar breaks down into two main substances: ethylic alcohol and carbon dioxide. There are traces of other chemical compounds, called secondary products, such as carboxylic acids, methanol, ether, aldehyde and superior alcohols (Vilela, Cardoso, Masson, & Anjos, 2007). Some of these products possess undesirable characteristics like formaldehyde, benzaldehyde, which has a narcotic effect, furfural, and ethyl carbamate (EC), which are probably carcinogenic (Labanca & Glória, 2006).

Ethyl carbamate, or urethane ($C_2H_5OCONH_2$, CAS No. 51-79-6), has several commercial uses, such as the preparation and modification of amino resins, as co-solvent for pesticides or manufactured drugs, and as a chemical intermediate in the textile industry to impart wash-and-wear properties (IRCA, 1974). In the past, EC was

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also used as an anti-neoplastic agent and for other medical purposes (Paterson, Handon, Thomas, & Watkinson, 1946), in particular the treatment of multiple myeloma (Holland et al., 1996). It was found to be toxic as early as the 1940s and was discovered to be carcinogenic in 1943 (Nettleship, Henshaw, & Meyer, 1943; Handow & Sexton, 1946). Ethyl carbamate was also used in human hypnosis and as an anaesthetic for laboratory animals. Nowadays, ethyl carbamate and other simple carbamates (phenyl, methyl or butyl) have some uses for research purposes only (Gotor, 1999).

Ethyl carbamate is genotoxic and carcinogenic in a number of species, including mice, rats, hamsters and monkeys, which suggests a probably carcinogenic risk to humans (Goyer & Cherian, 1995). It is absorbed rapidly and nearly completely from the gastro-intestinal tract and the skin (Neves et al., 2007).

Mackenzie, Cline, and Macdonald (1990) identified a series of precursors involved in EC formation in beverage production. Those precursors include copper cyanate, lactonitrile, isobutyraldehyde, cyanohydrin, anions of cyanate, and thiocyanate.

Ough (1976) showed that urea, citrulline, and carbamylphosphate can react with ethanol, producing EC in wine. They reported that the amino acid citrulline found in grapes is also an EC precursor, but not to the extent of urea. They concluded that urea, an intermediate product of yeast metabolism, is the most important precursor in wine and demonstrated that arginine is preferentially metabolised by yeast to produce urea. If urea is not used by yeast, it can react with alcohol during fermentation to form EC. These authors suggested that if wine originally contained high concentrations of nitrogenous nutrients, these would be metabolised by yeast before urea; more urea would remain in the medium after fermentation and become available to react, forming high concentrations of EC.

In the fermentation of sugar-cane juice, cyanide compounds are most important in the formation of ethyl carbamate. They are more important than they are in wine as they are present in higher concentration. There has been little research into EC formation in the fermentation of sugar-cane to obtain *cachaça*. The majority of research results have dealt with EC quantification in the commercial product or in samples directly obtained from *cachaça* producers. Two chemical pathways have been proposed as most likely in the formation of ethyl carbamate from cyanide. The first is based on complexing cyanide to Cu^{2+} followed by its oxidation to cyanogen, with a subsequent disproportionation of cyanide to cyanate; cyanate may react with ethanol to form ethyl carbamate (Guerain & Leblond, 1992). The second pathway is based on oxidation under UV light of unsaturated compounds present in alcoholic beverages, which produce free radicals (organic or hydroperoxides), which catalyse the oxidation of cyanide to cyanate; this may occur during storage of beverages (Guerain & Leblond, 1992). The factors influencing ethyl carbamate formation from cyanide are pH, light, ethanol content, temperature, vicinity of carbonyl groups in organic molecules, and concentration of Cu^{2+} ions in the beverage (Battaglia, Conacher, & Page, 1990; Riffikin, Wilson, Howie, & Müller, 1946). The reaction of proteins with ethanol, catalysed by Cu^{2+} ions, is also proposed as a way other than *via* CN for the formation of ethyl carbamate in spirits (Riffikin, Wilson, & Bringhurst, 1989).

Since December 1985, when Canada introduced limits for EC levels in alcoholic beverages, much academic and industrial research has been carried out on EC content in beverages. Additionally, rules have been established in other countries regulating the presence of EC (Faria & Pourchet Campos, 1989). Canada limited EC levels to 150 $\mu g\ L^{-1}$ in distilled beverages. After 1987, the USA's Food and Drug Administration (FDA) outlined several actions for alcoholic beverage and wine producers to reduce EC levels. According to the current Brazilian legislation (Brasil, 2005) the obligatory control of EC levels in spirits and *cachaça* will begin in 2010. In the European Community there are no harmonised maximum levels for ethyl carbamate. Commission Recommendation 2010/133/EU recommends that the Member States should monitor the levels of ethyl carbamate in stone fruit spirits and stone fruit marc spirits during the years 2010–2012 (EFSA, 2010).

Besides being a matter of public health, EC in concentrations beyond the limit permitted by different legislations could be a barrier to Brazilian exportation (Beattie & Polyblank, 1995; Guerain & Leblond, 1992; Jones, 1998). Given these concerns, this work aims to: (a) quantify the formation of ethyl carbamate in the fermentation process of sugar-cane juice, and in different distilled fractions and in the vinasse during *cachaça* production; (b) measure copper concentrations in sugar-cane juice and the distilled fractions and verify its correlation with EC production.

2. Materials and methods

In order to observe the effect of autochthonous inocula, samples were collected in three different fermentation reactors during the sugar-cane harvesting season from June to October, 2008. Samples were collected in June repetition 1 (early-harvest season), August repetition 2 (middle-harvest season) and October repetition 3 (late-harvest season). All analyses were made in triplicate.

3. Fermentation and distillation

The experiments were carried out in a traditional *cachaça* distillery. To 600 L of sugar-cane juice, at 16 °Brix, 200 L of inoculum were added; this inoculum is known as *pé de cuba*, i.e., obtained from previous sugar-cane fermentation by native biota. The fermentation process was conducted with no nutrient addition to the diluted sugar-cane juice. After 24 h, 600 L of the fermented juice (called wine) were distilled in a copper alembic heated by burning sugar-cane bagasse in a furnace (direct fire). The sugar-cane juice, wine and *cachaça* were analysed for ethyl carbamate content.

Samples were collected at time zero (unfermented sugar-cane juice) and after 6, 12, 18 and 24 h of fermentation. During distillation process, distilled samples were obtained according to the fractions for analytical purposes. Therefore, samples were collected from the head (4 and 8 L), the heart (10, 28, 48, 68, 88, 108, 128 L), and the tail (133, 138, 143, 148 L). At the end of the distillation process, after collecting the last sample of tail, vinasse, the distillation residue, was also sampled to quantify EC.

4. Sample analyses

4.1. Alcoholic content

The percentage of alcohol in samples was measured in °Gay Lussac (°GL = % volume), i.e., by taking 100 mL of the distillate in a measuring flask and using a densitometer calibrated at 20 °C. From this, the percentage of alcohol in *cachaça* was found by referring to standard tables.

4.2. Copper analysis

Twenty millilitres of each homogenised fraction were diluted in Milli-Q water to 50 mL and then underwent nitroperchloric digestion. Copper content was determined by inductively-coupled plasma atomic absorption spectrometry (ICP-AES) using a Perkin Elmer 3300 DV apparatus (Perker Elmer Corporation, Norwalk, CT). The instrumental operating conditions of the ICP-AES were 40 MHz frequency and a 374 lines mm^1 double diffraction net, working under the following conditions: generator: 1300 W, plasma gas flow 15 $L\ min^{-1}$, cone spray nebulizer pressure: 60 psi, integration mode: peak area of three points. The analysis was conducted at

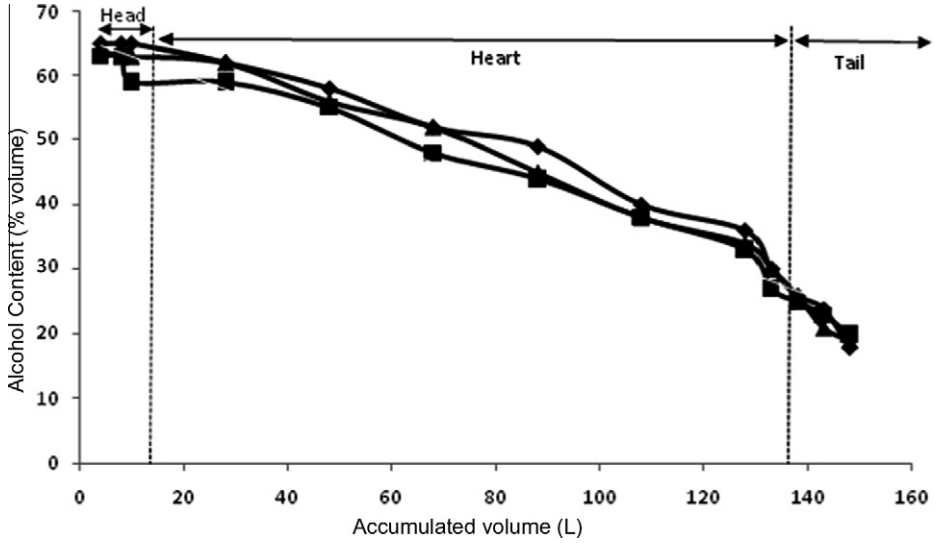

Fig. 1. Alcohol content as function of distillate collected during wine distillation (fermented cane juice) (■ repetition 1, ● repetition 2, ▲ repetition 3).

Table 1
Average copper content[b] in each fraction of *cachaça*.

Sample identification	Cu concentration (mg L^{-1})		
	1[a]	2[a]	3[a]
Sugar-cane juice	0.049	0.058	0.055
Head	8.68	8.85	8.75
Heart	2.97	3.33	3.11
Tail	5.28	5.15	5.12

[a] Repetitions.
[b] Determination of copper content was made after full collection of each fraction.

room temperature (20 °C) and detection at 327.4 nm. Detection limit of analysis was 0.009 mg kg^{-1} and method detection limit was 4.56 mg kg^{-1}.

4.3. Ethyl carbamate (EC) analysis

All samples collected during the fermentation process as well as the distilled fractions (head, heart, tail) and vinasse were analysed to determine EC. A Shimadzu GC 17-A gas chromatograph (Kyoto, Japan) was used with a Shimadzu QP-5050A mass spectrometer, in electron impact mode (70 eV). A DB-Wax column (60 m × 0.25 mm; 0.50 μm film thickness; Agilent, Santa Clara, CA) was used to separate EC. The temperature of injector and detector interface was maintained at 220 °C. The GC oven was programmed as follows: the initial temperature was 90 °C (2 min), then it was raised to 150 °C at a rate of 10 °C min^{-1}, then raised to 230 °C at a rate of 40 °C min^{-1}, and held for 10 min at this temperature. Injected volume was 2.0 μL (splitless). The carrier gas was helium (5.0) at a flow rate of 1.0 mL min^{-1}. The acquisition mode was SIM, monitoring ions *m*/*z* 62, 74 and 89.

Quantification was done by comparing chromatographic results of samples in an analytical curve obtained through an EC 99% solution (1.0 mg mL^{-1} in 40% ethanol; New Química) diluted to obtain a concentration range of 5–5000 μg L^{1}. Detection (LOD) and quantification (LOQ) limits of analysis were 15 and 50 μg L^{-1}, respectively.

5. Results and discussion

There was no observed difference between samples collected in each reactor used for chemical analysis in each repetition (June, August, October). Consequently, the end result of all analyses conducted was expressed as the average of three samples, obtained by repetition.

Fig. 1 shows the alcoholic content of samples analysed. It was possible to observe a regular pattern in alcoholic content between repetitions: up to 8 L (head) samples were approximately 65% (v/v) in alcohol; up to 128 L (heart) this content decreased to 35% (v/v) in alcohol; in the tail (133 at 148 L) the alcoholic content fell to less than 20% when distillation was stopped. After mixing collected fractions in the "heart" the final product (*cachaça*) possessed an alcoholic content of nearly 44% (v/v), in accordance with Brazilian law.

Table 1 shows the results of the copper analysis in the evaluated fractions. Brazilian legislation requires that its content in *cachaça* should be lower than 5.0 mg L^{-1} and it could be observed that the heart fraction was in accordance with the legislation. As shown in the table the head fractions did not fulfil the demands of legislation, and the copper content in the tail was very close to the legislation's limit. This result corroborates the need to separate head and tail from the heart fraction to ensure *cachaça* quality.

Cachaça is usually distilled in copper retorts and copper contamination can take place, as is confirmed by our results. The distillation in copper apparatus is, however, necessary to guarantee good sensorial properties in the product, due to the catalytic effects for the formation of flavour compounds (Neves et al., 2007). Comparative studies of volatile profiles of samples, distilled in the presence and absence of copper, revealed some differences, but the compounds related to this sensory defect were not identified (Faria et al., 2004). Faria (2003) showed that copper, when present in the distillation process, reduces the dimethyl sulphide (DMS) content; this may be mainly responsible for the characteristic sensory defect of *cachaça* distilled in the absence of copper. However, it is well known that copper has the adverse effect of catalysing the formation of ethyl carbamate.

GC–MS analyses of ethyl carbamate were performed by selected ion monitoring of *m*/*z* 62. The retention time of EC was between 13.4 and 13.6 min. During sugar-cane juice fermentation, EC values changed from zero in the sugar-cane juice to a maximum level of 160 mg L^{-1} in wine (after 24 h of fermentation) as shown in Fig. 2. Average EC value after fermentation period from three repetitions was 122 mg L^{-1}.

Sugar-cane (*Saccharum officinarum* L.), the raw material for Brazilian *cachaça*, is classified as a cyanogenic crop, but its cyanide

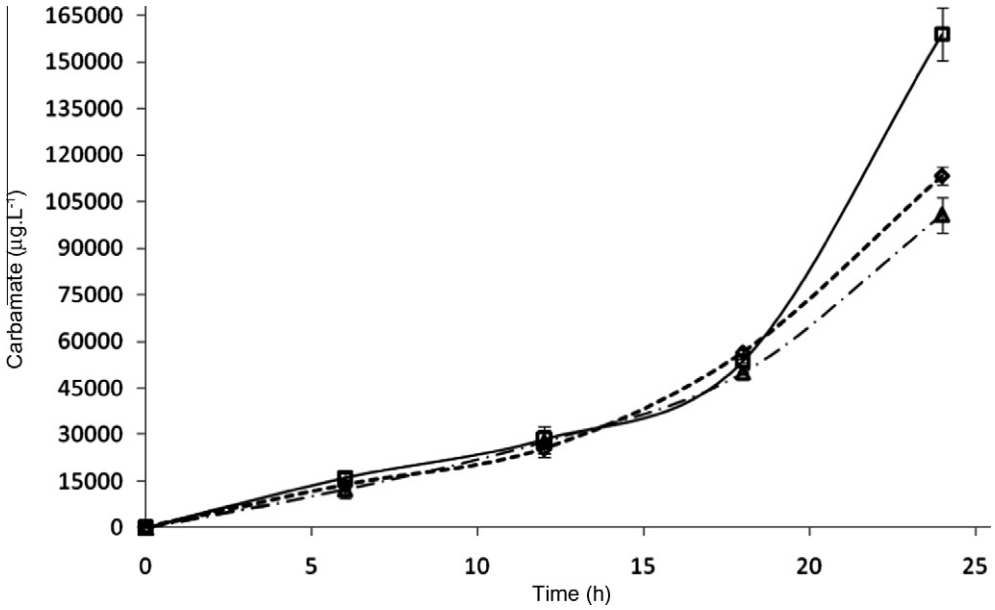

Fig. 2. Concentration of EC in cane juice during fermentation process to obtain *cachaça* (■ repetition 1, ● repetition 2, ▲ repetition 3).

Table 2
EC content (µg L^{-1}) in head and tail fractions.

Fraction/accumulate volume distilled	R1	R2	R3	Average
Head/4 L	7760	4710	4660	5710
Head/8 L	60,700	60,100	58,300	59,700
Heart/10 L	120	80	140	120
Heart/28 L	137	138	135	137
Heart/48 L	162	140	163	162
Heart/68 L	120	129	75.7	120
Heart/88 L	123	172	140	140
Heart/108 L	140	128	136	136
Heart/128 L	150	90.8	115	132.5
Tail/133 L	570	126	439	378
Tail/138 L	800	852	512	721
Tail/143 L	710	606	1160	825
Tail/148 L	1570	977	740	1096
Vinasse	55,300	51,300	52,600	53,100

source is as yet unknown (Beattie & Polyblank, 1995). As sugar-cane is poor in protein content and copper was present at low concentrations in sugar-cane juice (Table 1), these factors probably do not explain the quantity of EC formed. As shown in Fig. 2, there is EC formation during sugar-cane fermentation. Since no ingredient was added to the fermentation tank, these results suggest that EC production results from yeast metabolism. Carbamyl phosphate (CP) produced by yeast (*Saccharomyces cerevisiae*) can react with ethanol to generate ethyl carbamate in wine. CP comes from arginine, catalysed by carbamyl synthase, involving ATP, CO_2, and ammonia (Ingledew, Magnus, & Patterson, 1987). Intermediates such as carbamyl phosphate (CH_4NO_5P) are also easily formed *in vitro*. The results for EC content in the each fraction of distilled samples are shown in Table 2. for each repetition (R – June, August, October).

During distillation, the "head" is boiled off first. Components with low boiling point, e.g., ethyl acetate and methanol, are part of the "head". At the end of the first fraction collected (8 L), there was a high level of EC (average 59.7 mg L^{-1}), confirming that this fraction was unsuitable for consumption and must be discarded. Their use in subsequent distillations, as is the current practice of traditional *cachaça* producers must not be tolerated. These results showed the importance of separating the head fraction from the heart fraction. In direct-fire alembic the wine boiling temperature reaches 100 °C and alcoholic vapour exits over 90 °C, lower than EC boiling temperature, which is 186 °C (Neves et al., 2007). Despite these temperature conditions, EC is arrested during all distillation process. EC may be present in the head due to molecular interactions between ethanol and other chemical compounds present in the wine.

During the middle distillation run (the 'hearts'), the principal alcohol in all spirits, ethyl alcohol (ethanol), is distilled. This part of the distilling run, where the content of volatiles other than ethanol is lowest and the purest and typical aromas are present, is collected and sometimes aged. EC behaviour in this fraction can be seen in Table 2. It can be verified that EC content in the distilled portions remained under the limit of 150 µg L^{-1} in most samples. The observed variation probably occurs because of the alembic heating system, by burning bagasse which does not provide a constant rate of heat transference. The rate of heat transfer depends on the feeding frequency of the cane bagasse burning in the furnace.

When the alcohol content of the current distillate falls to 35% (v/v) in the fraction, collection has to be changed and the new fraction collected is known as 'tails'. In our case, this point occurs after collection of 128 L of distillate. The contents of the EC in this fraction increases. Composition of this fraction includes acetic acid and fusel oils, which are often identified by unpleasant vinegary and vegetal aromas (Boscolo, Bezerra, Cardoso, Lima-Neto, & Franco, 2000). They are also discarded. The EC average tail content was 1.10 mg L^{-1} and showed a continuous increase in concentration. The tail fraction showed a concentration of EC above the limit established by Brazilian law and the International Standard for distilled spirits. These results confirm the necessity to separate each fraction during production of cachaça.

Finally, in the last fraction, vinasse residue, an EC average concentration of 53.1 mg L^{-1} was found.

6. Conclusions

The results indicate that ethyl carbamate is formed during fermentation and its concentrations increases during distillation, corroborating the need to separate head and tail fractions to ensure

cachaça quality. It is essential to obey the limits of this compound established by legislation and even to avoid its presence in final product.

More studies are necessary to elucidate the pathway(s) involved in the formation of ethyl carbamate in fermented foods and beverages like *cachaça*.

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